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Hedgehog induction of murine vasculogenesis is mediated by Foxf1 and Bmp4

Jeanette Astorga and Peter Carlsson*

The first vasculature of the developing vertebrate embryo forms by assembly of endothelial cells into simple tubes from clusters of mesodermal angioblasts. Maturation of this vasculature involves remodeling, pruning and investment with mural cells. Hedgehog proteins are part of the instructive endodermal signal that triggers the assembly of the first primitive vessels in the mesoderm. We used a combination of genetic and in vitro culture methods to investigate the role of hedgehogs and their targets in murine extraembryonic vasculogenesis. We show that Bmps, in particular Bmp4, are crucial for vascular tube formation, that *Bmp4* expression in extraembryonic tissues requires the forkhead transcription factor Foxf1 and that the role of hedgehog proteins in this process is to activate *Foxf1* expression in the mesoderm. We show in the allantois that genetic disruption of hedgehog signaling (*Smo*$^{-/-}$) has no effect on *Foxf1* expression, and neither *Bmp4* expression nor vasculogenesis are disturbed. By contrast, targeted inactivation of *Foxf1* leads to loss of allantoic Bmp4 and vasculature. In vitro, the avascular *Foxf1*$^{-/-}$ phenotype can be rescued by exogenous Bmp4, and vasculogenesis in wild-type tissue can be blocked by the Bmp antagonist noggin. Hedgehogs are required for activation of *Foxf1*, *Bmp4* expression and vasculogenesis in the yolk sac. However, vasculogenesis in *Smo*$^{-/-}$ yolk sacs can be rescued by exogenous Bmp4, consistent with the notion that the role of hedgehog signaling in primary vascular tube formation is as an activator of *Bmp4*, via Foxf1.

KEY WORDS: Bmp, Forkhead, Hedgehog, Vasculogenesis, Mouse

INTRODUCTION

Our vasculature is a highly dynamic organ, constantly reorganized and modified to adjust to local changes in the need for oxygen and nutrients. The dominating mechanism of neovascularization in the adult is angiogenesis, i.e. extension or remodeling of existing vessels, usually by outgrowth of vascular sprouts. By contrast, the first embryonic vasculature is created through vasculogenesis, i.e. the formation of tubes of endothelial cells directly from mesodermal progenitors. Endothelial cells fuse into a primitive capillary plexus, from which the mature vasculature develops through remodeling, pruning and investment with pericytes and smooth muscle cells (SMCs). At the molecular level, adult neovascularization recapitulates many of the steps of embryonic vascular development (Bikfalvi and Bicknell, 2002; Jain, 2003).

Vascular endothelial growth factor (Vegf) and its receptor Flk1 (Kdr – Mouse Genome Informatics) are essential in all blood vessel development – angiogenesis as well as vasculogenesis – and genetic ablation of either receptor or ligand leads to a completely avascular phenotype [for a review of Vegf in vascular development, see Coultas et al. (Coultas et al., 2005)]. Angioblasts express Flk1 and require Vegf for proliferation, differentiation and survival. Vegf production is induced by hypoxia and concentration gradients of Vegf guide vascular sprouts into poorly oxygenated areas (Gerhardt et al., 2003). In vitro models and mutant phenotypes in several vertebrate species have illustrated the involvement of additional growth factor pathways in vascular development, but the relationships between them are not fully understood.

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Bone morphogenetic proteins (Bmps), in particular Bmp4, are required for mesoderm formation and consequently for development of all mesodermally derived tissues, including blood vessels (Mishina et al., 1995; Winnier et al., 1995). Bmp4 ventralizes mesoderm and is antagonized by dorsal/midline Bmp inhibitors such as noggin (Harland, 1994). In vitro, Bmp4 induces formation of Flk1^{+} Tal1^{+} cells, which require Vegf for proliferation and further differentiation (Park et al., 2004). In quail, ectopic Bmp4 has been shown to induce vascularization at the midline and expression of *Quek1* (the *Flk1* homolog) in the lateral plate (Reese et al., 2004). Interpretation of the phenotypes of loss-of-function mutants in mouse is complicated by the importance of Bmp signaling in gastrulation. However, *Bmp4*-null embryos that survive into the somite stage display a paucity of blood islands and extraembryonic mesoderm in the yolk sac (Winnier et al., 1995). Furthermore, targeted inactivation of either of the Bmp signal transducers Smad1 (Lechleider et al., 2001; Tremblay et al., 2001) or Smad5 (Chang et al., 1999; Yang et al., 1999) leads to embryonic lethality owing to a defective vasculature.

Several lines of evidence implicate the hedgehog pathway in vertebrate blood vessel formation. Inactivation of sonic hedgehog (*Shh*) is associated with decreased or defective vascular development (Brown et al., 2000; Pepicelli et al., 1998), whereas its overexpression in neuroectoderm causes hypervascularization (Rowitch et al., 1999). In adult mice, Shh stimulates neovascularization (Pola et al., 2001). In contrast to ectopic Vegf, which mainly induces endothelial sprouts, Shh promotes the entire angiogenesis program, including recruitment of mural cells, remodeling and vascular maturation (Pola et al., 2001). Extraembryonic vasculogenesis requires signals from the visceral endoderm and hedgehog proteins have been shown to be part of this signal. Indian hedgehog (*Ihh*) is highly expressed in the murine visceral endoderm (Becker et al., 1997; Farrington et al., 1997) and is the primary hedgehog family member responsible for induction of yolk sac vasculogenesis. Targeted inactivation of *Ihh* leads to

poor development of the yolk sac vasculature (Byrd et al., 2002), which results in the death of approximately 50% of *Ihh*-null embryos at mid-gestation (St-Jacques et al., 1999). $Ihh^{-/-}$ embryoid bodies are unable to form blood islands (Byrd et al., 2002) and recombinant Ihh can substitute for visceral endoderm as the inducer of vasculogenesis and activator of Bmp4 in tissue recombination experiments (Dyer et al., 2001). In chicken embryos, Shh can substitute for endoderm as the inducer of vascular tube formation (Vokes et al., 2004).

Different models have been put forward regarding the mechanism through which hedgehogs induce vascular development. In adult mice, Shh has been reported not to act directly on the endothelial cells, but rather through interstitial mesenchymal cells that respond by producing angiopoetins and Vegf (Pola et al., 2001). Vokes et al. (Vokes et al., 2004) suggested that Shh induces embryonic vascular tube formation by directly influencing the morphological properties of endothelial cells independently of Vegf.

Here, we use a combination of genetics and in vitro explant culture techniques to determine the mechanisms by which the hedgehog and Bmp signaling pathways regulate extraembryonic vascular tube formation. We show that *Foxf1* (also known as *Foxf1a* – Mouse Genome Informatics), which encodes a forkhead transcription factor, is a mesodermal target for endodermal hedgehog signaling. Foxf1 activates the expression of *Bmp4* in mesodermal cells, which in turn induces vascular tube formation. In one tissue undergoing extensive vasculogenesis – the murine allantois – expression of *Foxf1* is independent of hedgehog. Allantoic *Bmp4* expression and vasculogenesis are unaffected by abrogation of hedgehog signaling through deletion of smoothened (*Smo*). Genetic inactivation of *Foxf1*, on the other hand, leads to loss of both *Bmp4* expression and vasculogenesis in this tissue, a defect that can be rescued in vitro by exogenous Bmp4. That the same pathway also operates in a tissue where vasculogenesis normally requires hedgehog was shown by the ability of exogenous Bmp4 to rescue vascular plexus formation in $Smo^{-/-}$ yolk sacs.

MATERIALS AND METHODS

Mouse strains

The $Foxf1^{-}$ strain ($Foxf1^{tm1Pca}$) has been described elsewhere (Mahlapuu et al., 2001b). Shh^{tm1} (Chiang et al., 1996) was obtained from The Jackson Laboratory (Bar Harbor, ME). Ihh^{-} and Smo^{-} strains (St-Jacques et al., 1999; Zhang et al., 2001) were kindly provided by Dr A. P. McMahon and a strain carrying the $Bmp4^{lacZ}$ knock-in allele (Lawson et al., 1999) was kindly provided by Dr B. L. M. Hogan. Wild-type mice were C57Bl/6 (Charles River) and all mutants were maintained by breeding with this strain. Genotyping was performed by PCR as previously described (Lawson et al., 1999; Mahlapuu et al., 2001b; St-Jacques et al., 1999; Zhang et al., 2001).

In situ hybridization, immunohistochemistry and histological staining

Embryos heterozygous for the $Bmp4^{lacZ}$ allele were stained with X-Gal (Hogan et al., 1994), embedded in paraffin and sectioned. Whole-mount in situ hybridization was performed as described (Blixt et al., 2000) with an antisense RNA probe for *Foxf1* (Mahlapuu et al., 2001a). Rat monoclonal antibodies against Pecam (also known as Pecam1 – Mouse Genome Informatics) and Flk1 as well as a FITC-conjugated anti-CD41 (Itga2b – Mouse Genome Informatics) were purchased from BD Biosciences Pharmingen, and the FITC-conjugated anti-SMA (Acta2 – Mouse Genome Informatics) mouse monoclonal from Sigma. Detection of rat monoclonal antibodies was by biotinylated secondary antibodies and either HRP-streptavidin amplification with the TSA TM Biotin System (NEN Life Science Products) and DAB, or Alexa Fluor-streptavidin (Molecular Probes) immunofluorescence. Haematoxylin-Eosin (H&E) staining of paraffin sections was used for general histology.

Explant cultures

Allantoic buds were dissected from E8.25 embryos and cultured on cover slips for 24 hours in DMEM medium (Gibco) supplemented with 10% fetal calf serum, L-glutamine (2 mM) and penicillin-streptomycin (10 U/ml) in 5% CO_2, 100% humidity. Recombinant Bmp2, 4, 6 and 7 (0.1 ng/ml) and noggin (1 or 10 ng/ml) (all from R&D systems) were added to the culture medium when the explant cells had adhered to the substrate (approximately 3 hours after dissection). Yolk sacs were dissected and spread, mesoderm side up, on MF filters (Millipore), supported by stainless steel grids in 30 mm culture dishes. Approximately 3 ml of BGJb medium (Life Technologies) supplemented with 0.2 mg/ml ascorbic acid, 10 U/ml penicillin-streptomycin and 0.1% BSA was added to establish an air-fluid interface at the level of the explants and the cultures were kept at 37°C in 5% CO_2, 100% relative humidity for 24 hours. All results were verified by repeating the explant culture experiment at least three times.

RESULTS

Foxf1 expression and vasculogenesis are activated by Ihh and Shh

During organogenesis, *Foxf1* is activated in mesenchyme of the lung and gut in response to endodermal hedgehog signaling (Mahlapuu et al., 2001a; Ormestad et al., 2006). To investigate whether the same relationship exists in the early mouse embryo, we analyzed *Foxf1* expression in $Ihh^{-/-}$ embryos. Ihh is the dominating hedgehog in the yolk sac endoderm at the stage when the extraembryonic vasculature develops. At E9.5, $Ihh^{-/-}$ yolk sacs had fewer and thinner blood vessels, compared with wild-type (wt) (Fig. 1A,B) (Byrd et al., 2002). The reduced vasculature causes resorption of approximately 50% of *Ihh*-null embryos before establishment of a placental circulation, whereas the rest develop to term (St-Jacques et al., 1999). This contrasts with $Foxf1^{-/-}$ embryos, which have a fully penetrant avascular yolk sac phenotype (Fig. 1C) and are invariably resorbed by E10.5 (Mahlapuu et al., 2001b). Judged by whole-mount in situ hybridization, *Foxf1* expression was reduced in $Ihh^{-/-}$ blood islands, which at E8.5 appeared as a freckled staining pattern on the wt yolk sac surface (Fig. 1F,G). No alterations in expression pattern were observed in allantoic or lateral mesoderm (Fig. 1D,E).

The persistence of *Foxf1* mRNA in $Ihh^{-/-}$ embryos raised questions about whether, at this stage, *Foxf1* expression is independent of hedgehogs, or if other hedgehogs might compensate for the loss of Ihh. The severe phenotype of $Shh^{-/-}$; $Ihh^{-/-}$ embryos, as compared with either single mutant, indicates a widespread redundancy and a role for Ihh in processes previously thought to require only Shh (Zhang et al., 2001). Similarly, Shh might play a minor, but – in the absence of Ihh – significant, role in yolk sac development. We therefore analyzed $Shh^{-/-}$; $Ihh^{-/-}$ embryos and found their yolk sacs to be thin, transparent and essentially avascular (Fig. 2B,D). This indicates that Shh and Ihh both contribute to induction of yolk sac vasculogenesis. Closer inspection revealed adhesion between amnion and yolk sac in the hedgehog double mutants (arrowheads in Fig. 2B,D), which is reminiscent of the extensive adherences observed in extraembryonic mesoderm of *Foxf1* mutant embryos (Mahlapuu et al., 2001b). No *Foxf1* mRNA could be detected in yolk sac or lateral mesoderm of hedgehog double-null embryos. However, the allantois and the most-posterior embryonic mesoderm, exiting from the posterior primitive streak, expressed *Foxf1* (Fig. 2F).

The wrinkles on the E9.5 $Foxf1^{-/-}$ yolk sac surface (Fig. 1C) could be mistaken for blood vessels, but actually consist of folds in the visceral endoderm that are prevented from expanding by the constricted mesodermal layer (Fig. 3E,G) (Mahlapuu et al., 2001b). The yolk sac mesoderm in this mutant has abnormal adhesion properties, presumably owing to ectopic co-expression of Vcam1

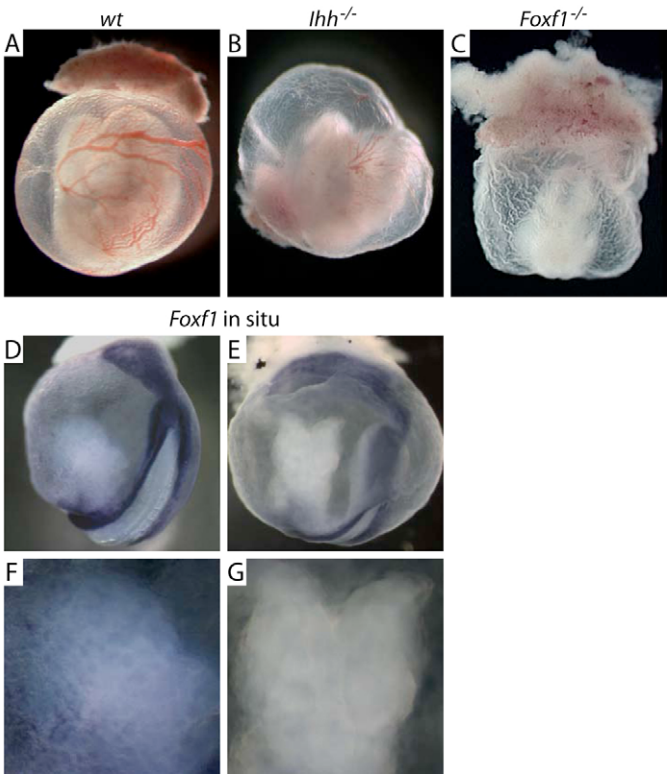

Fig. 1. Defective vasculogenesis in *Ihh*$^{-/-}$ yolk sac is associated with reduced *Foxf1* expression. (**A-C**) Comparison of yolk sac vasculature in E9.5 wild-type (wt) (A), *Ihh*$^{-/-}$ (B) and *Foxf1*$^{-/-}$ (C) mouse embryos. (**D-G**) Whole-mount in situ hybridization of E8.5 wt (D,F) and *Ihh*$^{-/-}$ (E,G) embryos with a *Foxf1* probe. F and G are magnified views of the yolk sac surface of the embryos in D and E, respectively. *Foxf1* expression in yolk sac blood islands is seen as diffuse blue speckles against the pale background of the out-of-focus embryo heads, and is much weaker in the *Ihh* mutant.

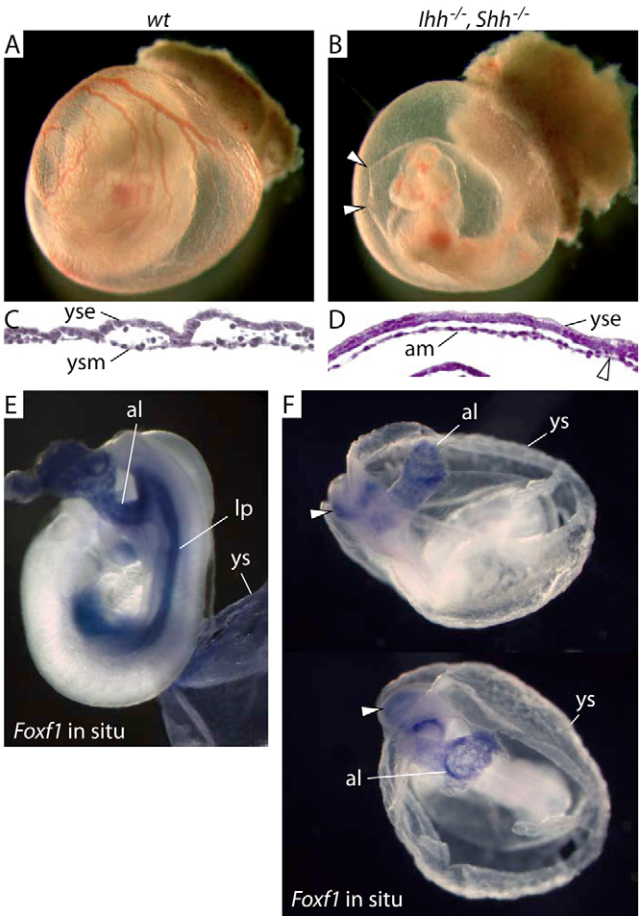

Fig. 2. Hedgehog signaling controls vasculogenesis and *Foxf1* expression. (**A-D**) Morphology and histology of wt and *Ihh*$^{-/-}$; *Shh*$^{-/-}$ E9.5 mouse embryos. (A) E9.5 wt embryo with well-developed yolk sac vasculature. The embryo has completed turning and reached the fetal position. (B) E9.5 *Ihh*$^{-/-}$; *Shh*$^{-/-}$ embryo with an almost completely avascular yolk sac. The embryo is still in the primitive, unturned position and the amnion adheres to the inner surface of the yolk sac (arrowheads in B). (C) Section of a wt E9.5 yolk sac with large blood vessels containing embryonic erythrocytes. (D) Section of an *Ihh*$^{-/-}$; *Shh*$^{-/-}$ E9.5 yolk sac. The yolk sac mesoderm consists of a thin lining of the inner surface of the yolk sac which in some areas adheres to the outer, mesodermal layer of the amnion (arrowhead). (**E**,**F**) Whole-mount in situ hybridization of E9.5 embryos with a *Foxf1* probe. (E) Wt embryo showing high-level expression of *Foxf1* in the lateral plate and extraembryonic mesoderm of allantois and yolk sac. (F) Lateral (top) and posterior-dorsal (bottom) views of an *Ihh*$^{-/-}$; *Shh*$^{-/-}$ embryo. *Foxf1* expression is absent in lateral mesoderm and yolk sac, but present in allantois and posterior primitive streak (arrowheads). al, allantois; am, amnion; lp, lateral plate mesoderm; ys, yolk sac; yse, yolk sac endoderm; ysm, yolk sac mesoderm.

with its ligand, α4-integrin, throughout the extraembryonic mesoderm (Mahlapuu et al., 2001b). The visceral endoderm was seen to be lined on the inner surface with a thin layer of endothelial (Pecam^{+} and Flk1^{+}; Fig. 3) cells, but the bulk of the yolk sac mesoderm was detached and formed a thick layer between the amnion and the yolk sac endoderm. The separation started in the mesometrial pole at E8.5 and spread to the entire yolk sac by E9.5 (Fig. 3E,G,I,J). The prolific and adhesive mesoderm of yolk sac and amnion sets the *Foxf1*$^{-/-}$ mutant apart from the *Shh*$^{-/-}$; *Ihh*$^{-/-}$ mutant, in which the amnion appeared normal and the yolk sac mesoderm was hypoplastic and reduced to a thin lining of the endoderm.

Hedgehog-independent *Foxf1* expression and vasculogenesis in the allantois

The residual *Foxf1* expression in *Shh*$^{-/-}$; *Ihh*$^{-/-}$ double knockouts could potentially be due to the third hedgehog family member, desert hedgehog (Dhh), or to hedgehog-independent mechanisms. In the case of the allantois, which at the analyzed stages had fused with the chorion to form the placenta, there is also the possibility of influence from maternal factors. Whole-mount in situ hybridization showed *Dhh* expression associated with the yolk sac vasculature at E10.5, but failed to detect any *Dhh* mRNA in the embryo proper or extraembryonic structures at E8.5 (see Fig. S1 in the supplementary material). This suggested that Dhh is not responsible for the activation of *Foxf1* in allantois and primitive streak, although we could not exclude the potential importance of an expression level below the detection limit. We therefore analyzed *Foxf1* expression in *Smo*$^{-/-}$ embryos. *Smo* encodes the signal-transducing component of the hedgehog receptor and its inactivation abrogates all hedgehog signaling (Zhang et al., 2001). The *Smo*$^{-/-}$ embryos were indistinguishable from *Shh*$^{-/-}$; *Ihh*$^{-/-}$ embryos and showed robust *Foxf1* expression in the allantois (Fig. 4C,D), as well as in the primitive streak region (Fig. 4D). Hence, *Foxf1* transcription in these

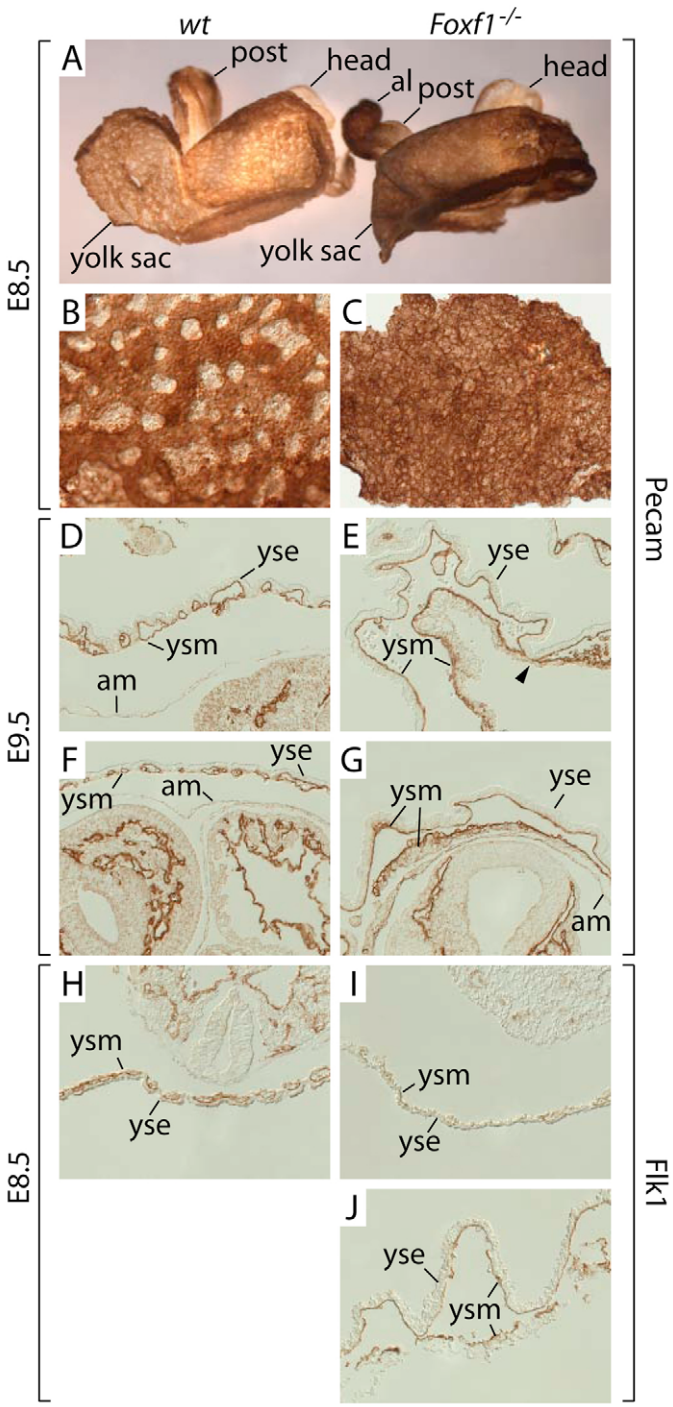

Fig. 3. Defective vasculogenesis in *Foxf1*$^{-/-}$ yolk sac. (**A-C**) Whole-mount Pecam staining of E8.5 wt (A left, B) and *Foxf1*$^{-/-}$ (A right, C) mouse embryos. B and C are higher magnifications of parts of the yolk sacs of the embryos in A. A primitive vascular plexus of endothelial tubes is well developed in the wt, but absent from the mutant. (**D-G**) Pecam staining of paraffin sections of E9.5 wt (D,F) and *Foxf1*$^{-/-}$ (E,G) embryos. In the *Foxf1* mutant, the yolk sac mesoderm is divided into a thin Pecam^{+} layer that lines the inner surface of the yolk sac endoderm, and a detached thick layer that only makes sporadic contact with the yolk sac endoderm (arrowhead in E). (**H-J**) Flk1 staining of cryosections of E8.5 wt (H) and *Foxf1*$^{-/-}$ (I,J) embryos. The separation of the yolk sac mesoderm has started in the mesometrial pole of the yolk sac (J), but not in the antimesometrial (I). al, allantois; am, amnion; post, posterior part of the embryo proper; yse, yolk sac endoderm; ysm, yolk sac mesoderm.

tissues differs from that in lateral and yolk sac mesoderm by being independent of hedgehog signaling. Cryptic *Dhh* expression, as well as maternal hedgehogs, can be dismissed as reasons for the persistent expression of *Foxf1* in *Shh*$^{-/-}$; *Ihh*$^{-/-}$ double knockouts.

The rudimentary yolk sac vasculature in *Smo*$^{-/-}$ and *Shh*$^{-/-}$; *Ihh*$^{-/-}$ mutants is consistent with an important role of hedgehogs in vascular development, as suggested in several studies (reviewed by Byrd and Grabel, 2004). The loss of yolk sac *Foxf1* expression in these mutants, together with the avascular extraembryonic phenotype of *Foxf1*$^{-/-}$ embryos, fit a model in which *Foxf1* is a mesodermal target that mediates the role of hedgehogs in blood vessel formation. However, the allantois is a highly vascularized tissue in which *Foxf1* expression is independent of *Smo*. We therefore asked whether vasculogenesis would proceed normally in the absence of hedgehog signaling in a tissue that expresses *Foxf1*, by analyzing allantoic vascular development. Explants of wt allantoic buds spontaneously formed a simple, but prolific, vascular plexus within 24 hours of culture, readily identified by immunostaining for the endothelial marker Pecam (Fig. 4E). Allantoic buds from *Smo*$^{-/-}$ embryos formed extensive networks of endothelial tubes, indistinguishable from those of wt explants (Fig. 4F). This demonstrated that formation of a vascular plexus can occur independently of hedgehog signaling. Allantoic bud explants from *Foxf1*$^{-/-}$ embryos contained Pecam^{+} cells, but these did not form vascular tube networks (Fig. 6E). Taken together, these results show that *Foxf1* is an important component of the vasculogenesis pathway and that *Foxf1* expression requires hedgehog signaling in some, but not all, tissues.

The vasculogenic activity of Foxf1 is mediated by Bmp4

Which target genes of Foxf1 mediate its vasculogenic activity? Several observations suggest *Bmp4* as a good candidate. Expression of *Bmp4* has been shown to be activated by Foxf proteins in some tissues (Mahlapuu et al., 2001b; Ormestad et al., 2006). Furthermore, Bmp4 is known to be associated with blood vessel formation in several systems (reviewed by Moser and Patterson, 2005). Several predictions follow from the hypothesis that *Bmp4* is a key target of Foxf1 that induces vasculogenesis: *Bmp4* should be expressed at the sites and stages of active vasculogenesis; it should exhibit reduced expression in avascular tissues of the hedgehog and *Foxf1* mutants; and the distinct vascular phenotypes of *Smo*$^{-/-}$ and *Foxf1*$^{-/-}$ embryos should be reflected in a corresponding difference in *Bmp4* expression. A *Bmp4*^{lacZ} knock-in allele (Lawson et al., 1999) allowed visualization of *Bmp4* transcription in thin tissues such as the yolk sac and amnion. Embryos heterozygous for this allele had *lacZ* staining in all extraembryonic mesoderm (yolk sac, amnion and allantois), the primitive streak, the lateral plate mesoderm and the heart (Fig. 4G,H and Fig. 5A-E). In the *Smo*$^{-/-}$ background, *Bmp4*^{lacZ} expression in yolk sac and lateral plate was reduced dramatically, but no significant change was observed in the primitive streak or allantois (Fig. 4I,J). In the *Foxf1*$^{-/-}$ background, *Bmp4*^{lacZ} expression was similarly reduced in yolk sac and lateral plate and remained high in the primitive streak. However, in contrast to *Smo*$^{-/-}$, the *Foxf1* mutant embryos exhibited a dramatic reduction of *Bmp4*^{lacZ} expression in the allantois (Fig. 5F-J). These results suggest that *Bmp4* expression during gastrulation is independent of Foxf1, but as the cells move out of the primitive streak to form the lateral or extraembryonic mesoderm, maintenance of *Bmp4* transcription requires Foxf1. A similar relationship appears to exist between *Foxf1* and hedgehogs, because expression of *Foxf1* in the primitive streak is hedgehog-independent, but its persistence in lateral and yolk sac mesoderm requires Smo and at least one of the

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ligands, Shh or Ihh. The exception is the allantois, which retains *Foxf1* and *Bmp4* expression even in the absence of hedgehog signaling.

If *Bmp4* is a key target of Foxf1 in vasculogenesis, inhibition of Bmp signaling should block vascular tube formation. To test this, we used the allantoic bud explant culture system. Inhibition of Bmp signaling in wt explants by the addition of noggin blocked formation of endothelial tubes (Fig. 6A-C), showing that Bmps are essential for vasculogenesis in this tissue. Next, we reasoned that if the failure of vasculogenesis in *Foxf1*$^{-/-}$ embryos was due mainly to the reduction in Bmp4 expression, then exogenous Bmp4 might be sufficient to overcome this block. *Foxf1*$^{-/-}$ explants contained Pecam^{+} cells, but they failed to organize into a defined network of tubes (Fig. 6E). Supplementing the culture medium of *Foxf1*$^{-/-}$ explants with Bmp4 to a final concentration of 0.1 ng/ml restored their ability to form vessels (Fig. 6F), demonstrating that a crucial function of Foxf1 in vasculogenesis is to drive expression of *Bmp4*. Similar results were obtained with Bmp2, 6 and 7 (see Fig. S2 in the supplementary material), suggesting that vascular tube formation is not dependent on a specific Bmp ligand. Concentrations of exogenous Bmp4 one order of magnitude higher, or lower, were less efficient at supporting vasculogenesis.

The results presented here show that hedgehog signaling is dispensable for vascular tube formation in the allantois and suggest that this is accomplished by uncoupling *Foxf1* – and thereby *Bmp4* – expression from the hedgehog pathway. We next asked whether the same downstream mechanisms operate in a tissue where hedgehog signaling is essential for vascular development. In other words, can the requirement for hedgehogs be bypassed by the addition of Bmp4? We cultured yolk sac explants from *Smo*$^{-/-}$ embryos in vitro and supplemented the medium with Bmp4. Yolk sacs from E9.5 *Smo*$^{-/-}$ embryos were cut in half and the left- and right-hand sides cultured on separate permeable membranes. Bmp4 was added to one of the halves and after 24 hours of culture the explants were stained with the Pecam antibody. As shown in Fig. 6G,H, addition of Bmp4 led to formation of a well-developed plexus of endothelial tubes in spite of the absence of functional hedgehog signal transduction. This demonstrates that hedgehog signaling is

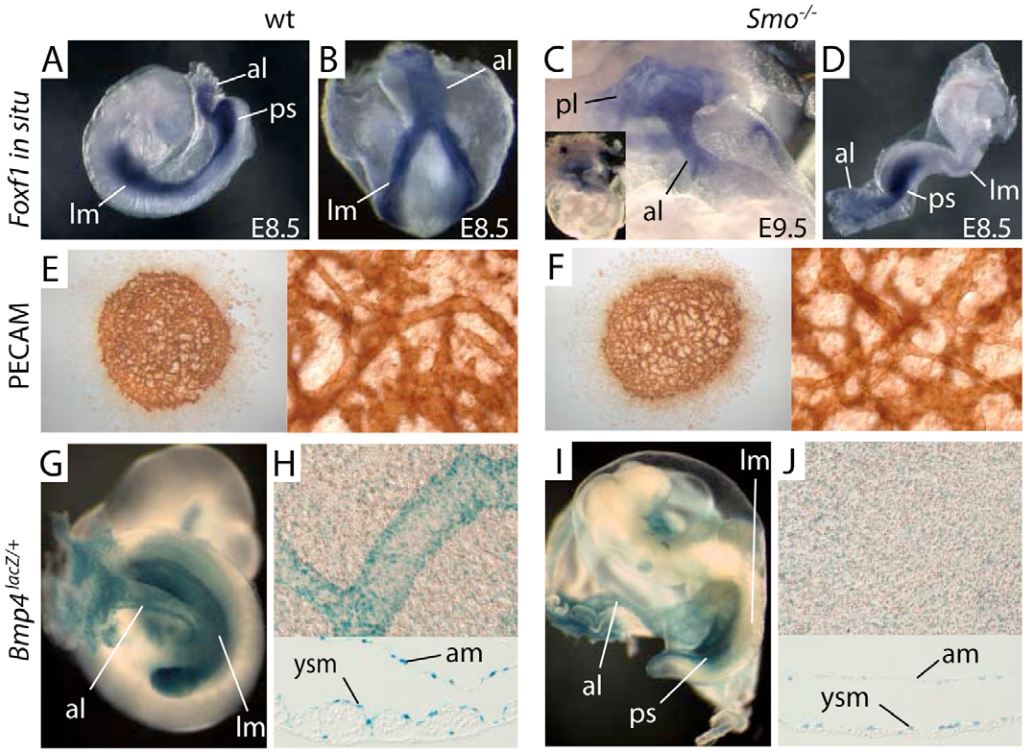

Fig. 4. The allantois undergoes normal vasculogenesis and maintains expression of *Foxf1* and *Bmp4* in the absence of hedgehog signaling. (**A-D**) *Foxf1* whole-mount in situ hybridization. (A) E8.5 wt littermate of the *Smo*$^{-/-}$ mouse embryo in D, showing *Foxf1* expression in lateral mesoderm, primitive streak and allantois. (B) *Foxf1* expression in lateral mesoderm, yolk sac and allantois of an E8.5 wt embryo still in the primitive, unturned position (shown for comparison with the unturned *Smo*$^{-/-}$ embryo). (C,D) *Smo*$^{-/-}$ embryos have the same pattern of *Foxf1* expression as *Ihh*$^{-/-}$; *Shh*$^{-/-}$ (see Fig. 2), i.e. in the allantois and primitive streak, but not in the lateral mesoderm or yolk sac. To show the chorioallantoic fusion, the entire E9.5 conceptus in C was left intact during hybridization. After the staining, a hole was dissected in the yolk sac through which the allantois and chorio-allantoic connection can be seen. *Foxf1* is expressed throughout the allantois, which emerges from the posterior end of the embryo and spreads out like a wide funnel over the chorionic surface (inset shows the entire yolk sac at low magnification). The E8.5 *Smo*$^{-/-}$ embryo in D has the yolk sac removed to show *Foxf1* expression in the primitive streak and its absence in the lateral mesoderm. (**E**,**F**) Pecam immunostaining of allantoic explants grown in vitro. Explants of unfused allantoic buds (E8.25) were grown for 24 hours, after which the primitive vascular plexus was visualized by immunostaining for the endothelial marker Pecam. Explants from *Smo*$^{-/-}$ embryos (F) developed a vasculature indistinguishable from that of wt embryos (E). (**G-J**) X-Gal staining of *Bmp4*^{lacZ} heterozygous E9.5 embryos homozygous for the wt (G,H) or null (I,J) alleles in the *Smo* locus. (G,I) *Bmp4*^{lacZ} expression in primitive streak and allantois is independent of hedgehog signaling, whereas expression in lateral mesoderm is lost in *Smo*$^{-/-}$. (H,J) Flat-mounted yolk sacs (top) and sections (bottom) showing *Bmp4*^{lacZ} expression in mesodermal cells of blood vessels. The avascular phenotype of *Smo*$^{-/-}$ yolk sac corresponds to reduced *Bmp4*^{lacZ} expression. al, allantois; am, amnion; lm, lateral mesoderm; pl, placenta; ps, primitive streak; ysm, yolk sac mesoderm.

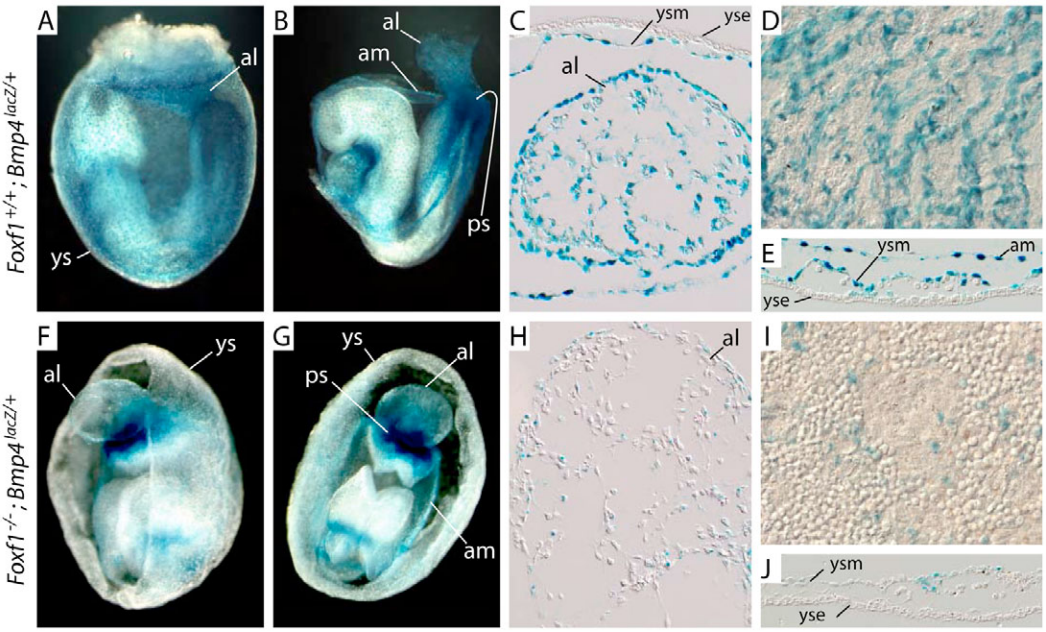

Fig. 5. Foxf1 is required for *Bmp4* expression in lateral and extraembryonic mesoderm. X-Gal staining was used to visualize expression from the *Bmp4*^{lacZ} allele in E8.5 *Foxf1*$^{+/+}$ (**A-E**) and *Foxf1*$^{-/-}$ (**F-J**) mouse embryos. (A) Intact wt conceptus with embryo that has just initiated turning. High *Bmp4* expression can be seen in both the allantois and yolk sac. (B) Unturned wt embryo with yolk sac removed showing *Bmp4* expression in the amnion (speckled pattern covering embryo), allantois, heart, and extending from the posterior primitive streak into the splanchnopleure and somatopleure. (C) Transverse section showing *Bmp4* expression in yolk sac mesoderm (at the top of the image), throughout the allantois and in the amnion (bottom). (D) The inner (mesodermal) surface of the yolk sac shows *Bmp4* expression associated with the developing vasculature. (E) Section showing *Bmp4* expression in yolk sac mesoderm and amnion. (F,G) Lateral (F) and dorsal (G) views of an *Foxf1*$^{-/-}$ embryo showing lack of *Bmp4* expression in the yolk sac and allantois. Only the primitive streak and heart retain high expression. (H) Section showing scattered and very weak *Bmp4* expression in *Foxf1*$^{-/-}$ allantois. (I) The inner (mesodermal) surface of a *Foxf1*$^{-/-}$ yolk sac shows only traces of *Bmp4* expression. (J) Section of *Foxf1*$^{-/-}$ yolk sac with characteristic separation between the endodermal and mesodermal layers. Occasional mesodermal cells express low levels of *Bmp4*. al, allantois; am, amnion; ps, primitive streak; ys, yolk sac; yse, yolk sac endoderm; ysm, yolk sac mesoderm.

dispensable for vasculogenesis in the yolk sac, as long as Bmp4 is present. It also proves that progenitors of endothelial cells are present, i.e. the *Smo*$^{-/-}$ yolk sac phenotype is not caused by a failure of progenitor cells to migrate and colonize the extraembryonic mesoderm.

Foxf1 and Bmp4 restrict allantoic smooth muscle cell differentiation

In addition to endothelial cells, the extraembryonic mesoderm gives rise to mesothelial, hematopoietic and smooth muscle cells. Previous results suggested that altered or mixed cell fates in *Foxf1*$^{-/-}$ extraembryonic mesoderm might contribute to the failure of vasculogenesis (Mahlapuu et al., 2001b). Primitive hematopoietic cells (ζ-globin^{+}) were aberrantly present in the amnion and abundant in the mesometrial pole of the yolk sac, but absent in the anti-mesometrial pole and in the allantois. Widespread misexpression of the SMC marker α-actin (SMA) was observed in yolk sac, amnion and allantois (Mahlapuu et al., 2001b). To investigate whether the excess of SMC progenitors was accompanied by a decrease in angioblasts, and whether this ratio is controlled by Bmp, we cultured *Foxf1*$^{-/-}$ allantoic explants in the presence and absence of Bmp4 and stained for SMA and Flk1 (Fig. 7). Wild-type explants had a moderate number of SMA^{+} cells loosely associated with the Flk1^{+} endothelial tubes (Fig. 7A). *Foxf1*$^{-/-}$ explants contained Flk1^{+} cells, but SMA^{+} cells were much more abundant and widespread in *Foxf1*$^{-/-}$ explants than in wt (Fig. 7B). Consistent with the presence of definitive hematopoietic progenitors in allatoic buds (Zeigler et al., 2006), a few CD41^{+} cells (Mikkola et al., 2003) were observed, but did not differ significantly in number between the genotypes (see Fig. S3 in the supplementary material). Inclusion of Bmp4 in the culture medium not only induced vascular formation, but also significantly reduced the number of SMA^{+} cells (Fig. 7C). Thus, Bmp can reverse the abnormal accumulation of SMA^{+} cells that results from inactivation of *Foxf1* and normalize the SMC/angioblast ratio. This might reflect an ability of Bmp signaling to influence fate decisions in the extraembryonic mesoderm, or to inhibit proliferation of SMC progenitors.

DISCUSSION

We present evidence for a pathway in which endodermal hedgehog ligands (Ihh and Shh) induce expression of *Foxf1* in the mesoderm; Foxf1 in turn activates *Bmp4* transcription and Bmp signaling promotes the assembly of vascular tubes from mesodermal progenitors (Fig. 8). In the presence of other sources of Bmp, neither hedgehog nor Foxf1 was required for vasculogenesis. For example, *Bmp4* expression in the heart was not affected by targeted inactivation of *Foxf1* and the endocardium developed normally in *Foxf1*$^{-/-}$ embryos. The allantoic vascular plexus developed normally in *Smo*$^{-/-}$ embryos. In both of these examples, endothelial tube formation was associated with robust *Bmp4* expression. However, apart from the primary assembly of endothelial tubes discussed here, development of a mature vasculature involves many additional steps and we do not exclude the possibility that hedgehogs and Foxf1 might have important functions in these later stages.

DEVELOPMENT

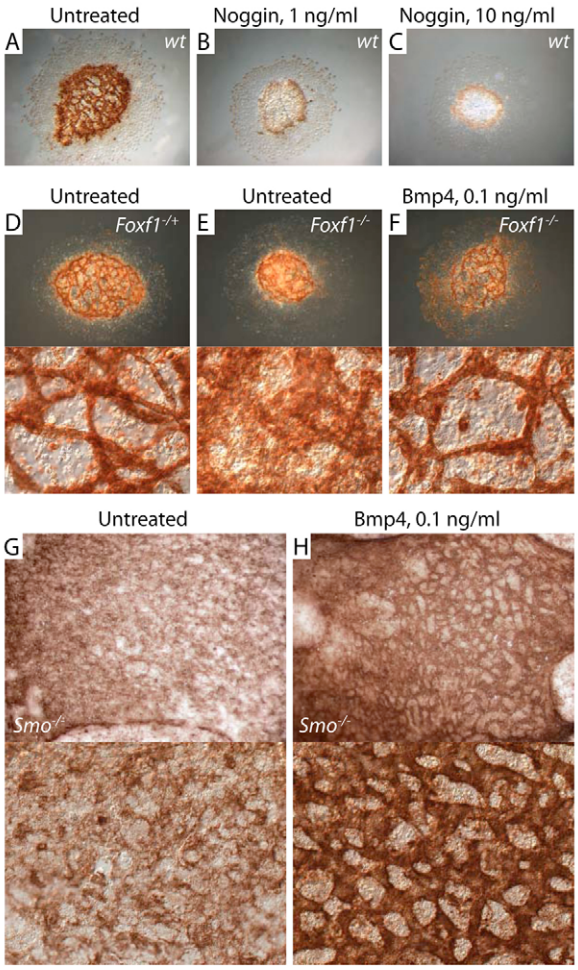

Fig. 6. Bmp signaling is essential for blood vessel formation and is a mediator of the vasculogenic activity of Foxf1. Explants of E8.25 allantoic buds (**A-F**) and *Smo*$^{-/-}$ E9.5 yolk sacs (**G**,**H**) were cultured in vitro for 24 hours followed by staining with Pecam antibody to visualize the capillary network. (A-C) Allantoic explants from wt mouse embryos grown in the absence (A) or presence (B,C) of the Bmp antagonist noggin at 1 (B) or 10 (C) ng/ml. (D-F) Rescue of blood vessel formation in *Foxf1*$^{-/-}$ explants by exogenous Bmp4. (D) Normal vasculogenesis in *Foxf1*$^{-/+}$ explant. (E) *Foxf1*$^{-/-}$ allantoic cells express Pecam, but fail to form a distinct vascular plexus. (F) Addition of 0.1 ng/ml Bmp4 to *Foxf1*$^{-/-}$ explants restored formation of the capillary network. (G,H) The yolk sac from an E9.5 *Smo*$^{-/-}$ embryo was dissected, cut in half and left- and right-hand sides spread on separate filters with (H) or without (G) Bmp4 (0.1 ng/ml) in the medium. After 24 hours in culture, explants were fixed and stained with Pecam antibody. Low-magnification images (top) show the entire explants and higher magnification views (bottom) show the formation of a vascular plexus in response to Bmp4.

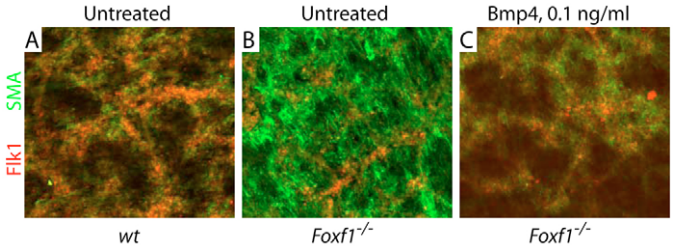

Fig. 7. Bmp4 restricts allantoic smooth muscle cell differentiation. Immunofluorescence showing Flk1^{+} (red) and SMA^{+} (green) cells in wt (**A**) and *Foxf1*$^{-/-}$ (**B**,**C**) allantoic explants cultured for 24 hours with (C) or without (A,B) Bmp4 (0.1 ng/ml).

The described pathway simplifies the interpretation of several observations that have troubled previously suggested models of direct, cell-autonomous involvement of hedgehog in vascular tube formation. For example, *Smo*$^{-/-}$ mutants have absent or severely defective dorsal aortae along much of the embryo, but towards the posterior end the morphology of these blood vessels improves to near normal (Vokes et al., 2004; Zhang et al., 2001). Inactivation of *Smo* abrogates all hedgehog signaling, which makes this gradient in vascular development difficult to reconcile with hedgehog being required for endothelial tube formation by acting directly on angioblasts. This prompted speculation of alternative pathways, both hedgehog-dependent and -independent, acting in different parts of the embryo (Byrd and Grabel, 2004; Vokes et al., 2004). The high level of hedgehog-independent *Bmp4* expression in the primitive streak area generates a localized, posterior source of Bmp4 in *Smo*$^{-/-}$ embryos, which readily explains the observed gradient in dorsal aorta development. In contrast to hedgehog signaling, which is completely blocked in *Smo*$^{-/-}$ mutants, Bmp4 expression is only reduced. Variation, stochastic or genetic, in the residual amounts of Bmp will inevitably give rise to interindividual phenotypic variation. This is seen, for example, in the development of *Smo*$^{-/-}$ embryonic vasculature (Vokes et al., 2004) and in the yolk sac of *Smo*$^{-/-}$ embryos, which often have patches of rudimentary plexus formation in the area closest to the embryo (Byrd et al., 2002).

Proliferation of primitive streak mesoderm is reduced in *Foxf1*$^{-/-}$ mutants, but no significant decrease in proliferation or survival was detected in the extraembryonic mesoderm (Mahlapuu et al., 2001b). This is consistent with the robust growth of *Foxf1*$^{-/-}$ allantoic explants in vitro and implies that the failure to form a vascular plexus is not a consequence of a general hypoplasia. In other systems, Bmps have been shown to increase Vegf expression and stimulate proliferation of Flk1^{+} cells (Deckers et al., 2002; He and Chen, 2005; Nimmagadda et al., 2005; Reese et al., 2004). It is therefore likely that Bmp acts through the Vegf pathway also in extraembryonic vasculogenesis.

Why does the allantois differ from other extraembryonic mesodermal structures with regard to activation of *Foxf1*? In fish and amphibians, vasculogenesis and hematopoiesis are initiated in ventral mesoderm. A vascular bed encloses yolk contained in the primitive gut and both hedgehog, expressed in the endoderm, and BMP4, expressed in the ventral mesoderm, are important for its formation (Brown et al., 2000; Harland, 1994). As an adaptation to terrestrial development, the endoderm-mesoderm bilayer of oviparous amniotes folds into three distinct compartments – the gut, the yolk sac and the allantois – all with the same basic organization and with vasculogenesis occurring throughout. The selective forces that shaped these extraembryonic structures are absent in placental mammals. Owing to phylogenetic constraints, the structures are still part of mammalian embryology, but have lost their original functions and in some cases acquired novel ones. In consequence, they display a remarkable morphological diversity across mammalian species. The murine allantois represents an evolutionary oddity that has lost the endodermal component and thus the source of hedgehog. This would arrest vasculogenesis, unless replaced by autocrine hedgehog signaling in the mesoderm, or by other means of activating the key

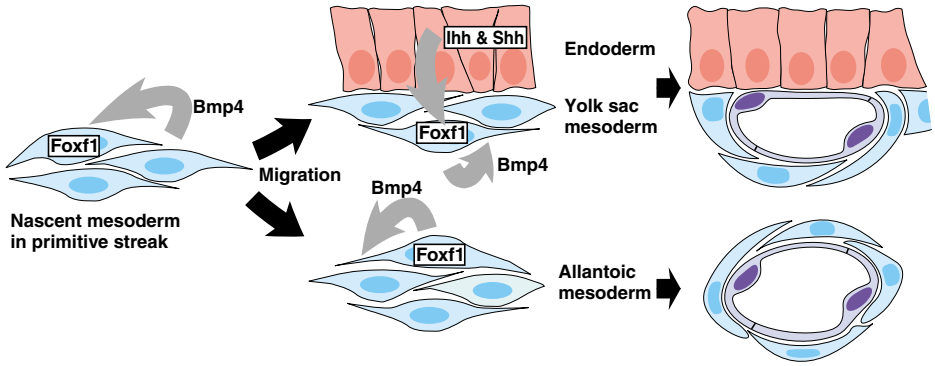

Fig. 8. Schematic summary of relationships between hedgehogs, Foxf1 and Bmp4 in different tissues. Expression of *Bmp4* precedes that of *Foxf1* in mesoderm formed in the primitive streak area during gastrulation; data from *Xenopus* (Tseng et al., 2004) indicate that Bmp4 activates transcription of *Foxf1* at this stage. As the posterior mesoderm migrates out of the primitive streak and associates with the endoderm in the lateral plate and yolk sac, maintenance of *Foxf1* expression depends on hedgehog ligands (Ihh and Shh) secreted by the endoderm. In the murine allantois, which lacks endoderm, *Foxf1* expression remains high in the absence of hedgehog signaling. Maintenance of robust *Bmp4* expression in the mesodermal cells, after exit from the primitive streak, requires Foxf1. Bmp4 induces the formation of endothelial tubes from mesodermal progenitors.

mesodermal targets. In mouse, this problem appears to have been solved by hedgehog-independent transcriptional activation of the *Foxf1* gene.

Speculation regarding redundancy between hedgehog paralogs as the explanation for the residual vasculature in *Ihh* mutants has focused on Dhh. *Dhh* is expressed in the yolk sac mesoderm, but has not been detected earlier than E10.5 (Farrington et al., 1997). *Shh* mRNA is present in the yolk sac endoderm, but at a low level that has been assumed to be without physiological relevance (Farrington et al., 1997). The phenotypes of $Shh^{-/-}$; $Ihh^{-/-}$ and *Smo* mutants were indistinguishable and differed strikingly from both hedgehog single nulls. This demonstrates a widespread redundancy between *Shh* and *Ihh*. Specifically, our results show that Shh contributes to yolk sac vascular development, but does not support the notion of a role for Dhh in this process. It should be emphasized that the main argument for dismissal of Dhh is not the absence of detectable expression, but the identical phenotypes (including allantoic vasculogenesis) of $Smo^{-/-}$ and $Shh^{-/-}$; $Ihh^{-/-}$.

Bmp4 expression during gastrulation does not require Foxf1. However, once mesodermal cells exit from the primitive streak and reach the lateral plate or extraembryonic structures, Foxf1 becomes essential for maintaining high-level *Bmp4* transcription. Injection of *BMP4* mRNA in 8-cell *Xenopus* embryos induced transcription of *FoxF1* in gastrula stage animal caps (Tseng et al., 2004). This is consistent with the onset of expression being earlier for *Bmp4* than *Foxf1*. Hence, during gastrulation, *FoxF1* expression in nascent mesoderm appears to require Bmp, whereas later on the relationship is reversed. Interestingly, a similar relationship has been described in *Drosophila* where Dpp (the Bmp homolog) is required for activation of *biniou* (the *Foxf* homolog) early in development in the trunk visceral mesoderm primordium, but Biniou activates *dpp* at later stages in the visceral mesoderm (Zaffran et al., 2001).

The activation of *FoxF1* by BMP4 in early *Xenopus* embryos (Tseng et al., 2004) contrasts with the inhibition of *Foxf1* in murine lung mesenchyme by Bmp4 from the distal lung bud epithelium (Mahlapuu et al., 2001a). The epithelio-mesenchymal cross-talk during lung branching morphogenesis is complex and it is unclear whether the effect on *Foxf1* is direct or indirect. In the early embryo, where *Foxf1* and *Bmp4* are co-expressed in the mesoderm, there is no evidence for feedback inhibition.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/20/4432/DC1

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